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Orchestration of carbohydrate processing for crassulacean acid metabolism

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Abstract

The production of phosphoenolpyruvate as a substrate for nocturnal CO₂ uptake represents a significant sink for carbohydrate in CAM plants which has to be balanced with the provisioning of carbohydrate for growth and maintenance. In starch-storing CAM species, diversification in chloroplast metabolite transporters, and the deployment of both phosphorolytic and hydrolytic routes of starch degradation accommodate a division of labour in directing C-skeletons towards nocturnal carboxylation or production of sucrose for growth. In soluble-sugar storing CAM plants, the vacuole plays a central role in managing carbon homeostasis. The molecular identities of various types of vacuolar sugar transporters have only been identified for C₃ species within the last 10 years. The recent availability of CAM genomes enables the identification of putative orthologues of vacuolar sugar transporters which represent strategic targets for orchestrating the diel provisioning of substrate for nocturnal carboxylation and growth.

Introduction

In plants with the photosynthetic specialisation of crassulacean acid metabolism (CAM), stomatal opening and primary CO₂ uptake occur at night when leaf-air vapour pressure deficit is low relative to that during the day. These water-conserving traits have identified CAM as a target for synthetic biology to engineer improved water use efficiency (WUE) into non-CAM crops as a means of sustaining productivity in the warmer and drier world predicted by global climate models [1,2]. The nocturnal CO₂ uptake that defines CAM is sustained by a dedicated pool of carbohydrate that is degraded each night to provide the 3-C substrate phosphoenolpyruvate (PEP) for PEP carboxylase (PEPC), the enzyme responsible for dark CO₂ uptake. The close stoichiometric relationship between nocturnal carbohydrate degradation and the magnitude of net CO₂ uptake at night indicates that carbohydrate availability is a key limiting factor for CAM [3-5]. At the same time, the day-time recovery and partitioning of carbohydrate for the nocturnal production of PEP must be balanced with the need to provision sucrose for growth, export and maintenance processes during the day and/or night [6]. Ultimately, the transporters and enzymes that regulate the provisioning of carbohydrates between nocturnal carboxylation, growth and maintenance dictate the magnitude of net carbon gain, productivity and WUE of CAM plants. Elucidating the mechanisms which orchestrate diel carbohydrate processing in CAM plants will be central for

ensuring that the bioengineering of CAM to improve plant WUE does not compromise plant productivity.

Depending upon the plant species, starch and/or soluble sugars can be used as the transitory store of carbohydrate that is degraded each night to sustain dark CO₂ uptake in plants where CAM is constitutively expressed or in facultative CAM plants where the pathway is induced in response to stressors such as drought or salinity [7,8]. PEP generated from nocturnal carbohydrate degradation combines with atmospheric and/or respiratory CO₂ to form oxaloacetate which is then converted to malic acid for storage in the vacuole (Figure 1). The next day, malate exits the vacuole and decarboxylation releases CO₂ which is re-fixed by Rubisco and processed by the photosynthetic carbon reduction cycle (PCR) whilst stomata remain closed and transpirational water loss is curtailed [9]. Carbohydrates are recovered from re-fixed CO₂ via the production of triose-P and hexose-P and from the gluconeogenic processing of PEP and/or pyruvate released from the day-time breakdown of malate [9]. Here, we review the pathways purported to underpin the processing of carbohydrates for nocturnal carboxylation and growth in CAM plants. Chloroplastic and vacuolar transporters are highlighted as potential strategic checkpoints for regulating the provisioning of carbohydrate for nocturnal carboxylation and growth. We also discuss how elements supporting the diel regulation of carbohydrate partitioning in CAM might have been derived from C₃ plants.

Diversity in chloroplast metabolite transporters accommodates balanced partitioning of carbohydrate between nocturnal carboxylation and growth

Chloroplasts of the starch-storing facultative CAM species *Mesembryanthemum crystallinum* contain transport systems for triose phosphate (TP), glucose-6-phosphate (GPT), glucose (pGlcT) and maltose (MEX) [10-12]. A division of labour among these transporters for directing carbohydrate towards nocturnal carboxylation or production of sucrose for growth was first suggested by the finding that chloroplasts isolated from C₃-performing *M. crystallinum* exported mainly maltose whilst chloroplasts isolated from plants in CAM exported predominantly Glc6P [10,13]. It is now known that induction of CAM in *M. crystallinum* is accompanied by a substantial increase in chloroplastic Glc6P transport rates [13] and a > 70 fold increase in transcript abundance of GPT whilst expression of the genes encoding TP or the plastidic glucose transporter (pGlc) remain relatively unchanged [11,14]. These findings support the hypothesis that the TP and pGlc transporters support the synthesis of sucrose in both C₃ and CAM modes by exporting triose-P during the day (TP) or glucose at night (pGlc) whilst the GPT assumes a CAM-specific role (Figure 1a).

Two genes encoding GPT are expressed in leaves of *M. crystallinum* and in pineapple, a CAM species which can use either starch or soluble sugars as substrate for the nocturnal provision of PEP [15], (Table 1). Robust diel rhythms of transcript abundance are observed for both *GPTs* in CAM-performing *M. crystallinum* [16,17] and pineapple. In *M. crystallinum*, transcript abundances of *GPTs* and transport activity of GPT increases when the rate of starch biosynthesis reaches a maximum around the middle of the photoperiod, suggesting that Glc6P is imported to the chloroplast during the day to enhance starch biosynthesis [11], (Figure 1). GPT transport activity tracks transcript abundance of *GPTs* which are under circadian control in *M. crystallinum* [16] suggesting that Glc6P import for starch biosynthesis is regulated by the clock. Such a mechanism could set a threshold potential for nocturnal CO₂ uptake by ensuring that plants enter the dark period with an adequate store of starch, irrespective of environmental conditions [3,18,19].

Persistence of *GPT1* and *2* transcripts over the first part of the dark period in *M. crystallinum* also indicates export of Glc6P derived from nocturnal starch degradation during early night when Glc6P accumulates in the chloroplast to high concentrations [11]. In pineapple,

transcripts are more abundant at night for the more highly expressed *GPT1*, compared to the day-time pattern for *GPT2*, implying a division of labour between *GPT* isogenes in directing nocturnal export or day-time import of Glc6P across the chloroplast envelope (Table 1).

The route of starch degradation is a key point of divergence between C₃ and CAM

A role for GPT in nocturnal provision of substrate for PEP production hinges on production of glucose-P via the phosphorolytic route of starch degradation which is catalysed by chloroplastic α -glucan phosphorylase (PHS1; [20]. In C₃ plants, starch is broken down at night via the hydrolytic route which produces maltose and glucose for export from the chloroplast; reviewed in [21,22]. In contrast, phosphorolytic starch degradation mediated via PHS1 in C₃ plants is believed to primarily provide substrate for internal chloroplast metabolism [23,24]. For CAM plants, phosphorolytic starch degradation and export of Glc6P from the chloroplast offers energetic advantages over hydrolytic degradation since glycolytic conversion of Glc6P to PEP in the cytosol provides ATP by substrate-level phosphorylation, thus helping to energise nocturnal vacuolar accumulation of malate [25,26]. Furthermore, Glc6P acts as an allosteric activator of PEPC [27] stimulating nocturnal CO₂ uptake and potentially coordinating nocturnal supply and demand for PEP.

Efforts are ongoing to establish what, if any, contribution the hydrolytic pathway makes to nocturnal starch degradation in CAM plants. A recent comparison of enzymatic activities in *Arabidopsis thaliana* and *M. crystallinum* operating in both C₃ and CAM modes indicated low *in vitro* activity in *M. crystallinum* of the cytosolic disproportionating enzyme (DPE2; Table 2), an enzyme crucial for the processing of exported maltose to sucrose in *A. thaliana* [28]. Low DPE2 activity could represent a bottleneck for production and export of maltose in the CAM species. In marked contrast, the *in vitro* activity of chloroplastic disproportionating enzyme (DPE1) is almost 5 fold higher in C₃-performing *M. crystallinum* and 10 fold higher when the plant is in CAM mode compared to *A. thaliana* (Table 2). In *A. thaliana*, DPE1 acts on maltotriose produced from hydrolytic starch breakdown in the chloroplast to generate glucose for export via the pGlc as well as maltopentaose for subsequent metabolism by β -amylase and PHS1 [29]. Greater flux through the pGLC compared to MEX is also supported by the greater transcript abundance of *pGLC* compared to *MEX1* in several CAM species, including pineapple (Table 1). These data, alongside on-going research on genetically modified lines of *Kalanchoe fedstchenkoi*, support the notion that phosphorolytic degradation of starch produces substrate for production of PEP whilst the hydrolytic production and nocturnal export of glucose via the pGlc provides substrate for PEP but also for sucrose synthesis (J Ceusters, J Hartwell, A Borland, unpublished observations; Figure 1a).

The emerging picture for starch degradation in CAM plants raises the question of how this re-routing of chloroplastic starch metabolism evolved from C₃? Chloroplastic PHS1 activity in *Arabidopsis* is thought to be particularly important under conditions of stress to supply substrates for the plastidial oxidative pentose phosphate pathway, potentially generating reductant (NADPH) for the scavenging of reactive oxygen species [23]. In *Arabidopsis*, the products of phosphorolytic starch degradation are not exported from the chloroplast because envelope GPTs are not generally expressed in mature photosynthetic tissue [30,31]. However, the expression of *GPT2* in *Arabidopsis* appears to be highly variable and sensitive to environmental stress [32], perturbed redox poise, [33,34], or situations when carbohydrate metabolism is impaired [35]. It is conceivable that the multiple evolutionary emergences of CAM from C₃ in typically extreme environments exploited the stress responsive up-regulation of PHS1 [14,36] and GPT [14]. In addition to energetic advantages from phosphorolytic starch breakdown [26], degradation of leaf starch by both phosphorolytic and hydrolytic routes in CAM plants offers the possibility for multiple levels of control that span source leaf activity and sink demand. Thus, the re-routing of

starch degradation in CAM ensures an appropriate provisioning of carbohydrate between nocturnal carboxylation and growth over the diel cycle and throughout plant development.

Vacuolar sugar transport and provision of carbohydrate for nocturnal carboxylation

For CAM species that include pineapple and *Agave*, soluble sugars sequestered in the central vacuole provide some or all of the PEP required for nocturnal carboxylation [15]. Vacuolar sugar transporters responsible for day-time import of sucrose and nocturnal export of sugars likely play important roles in regulating supply and demand for carbohydrate over the diel CAM cycle [37], (Figure 1b). The recently published pineapple genome [38] reveals several putative orthologues of vacuolar transporters expressed in leaves [39], (Table 1). The most abundant transcripts in the pineapple leaf are those encoding orthologues of the tonoplast sugar transporters (*TST*), [38]. In the C_3 species examined to date, *TST1* and *TST2* mediate the uptake of glucose, fructose [40] or sucrose [41] into the vacuole in counter exchange with protons. Although localisation studies are required to confidently confirm tonoplast localisation of these *TST* orthologues in pineapple, a *TST* orthologue has been identified using a proteomics analysis of tonoplast enriched membrane prepared from the leaves of *A. americana* (D. Al-Baijan, PhD Thesis, Newcastle University, 2015). Substrate specificity and transport mechanism of the *TSTs* in CAM species require confirmation, but on the grounds of conserved evolutionary function and parsimony between C_3 and CAM, the *TST(s)* could transport sucrose into the pineapple vacuole during the day in counter-exchange with H^+ . Exceptionally high activities of vacuolar ATPase have been reported for pineapple compared to C_3 and other CAM species [42]. Thus, transport capacity of vacuolar proton pumps in pineapple should be sufficient to sustain the necessary electrochemical gradient across the tonoplast in the face of H^+ linked sugar transport and the diel fluxes of malic acid that characterise CAM. Previous studies with tonoplast enriched vesicles showed that an H^+ gradient was not required for vacuolar sucrose uptake in pineapple [42]. High vacuolar invertase activity in pineapple [43] implies that once in the vacuolar lumen, sucrose would be hydrolysed to glucose and fructose which could sustain an inwardly directed gradient of sucrose influx from the cytosol. A similar scenario could operate in *Agave* where sucrose imported to the vacuole is converted to fructan [44]. It remains to be established if *TST* orthologues in pineapple and *Agave* function as uniporters or proton-antiporters.

Putative orthologues of several vacuolar sugar exporters have also been identified in the pineapple leaf transcriptome (Table 1); a sucrose exporter (*SUC4*, [45], a glucose exporter *early response to dehydration like 6*, (*ERDL6*: [46]), and *SWEET16*, a putative vacuolar member of the *SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERS* which in *Arabidopsis* operate in the facilitated diffusion of fructose, glucose or sucrose [47]. *ERDL6* was previously shown to be localised to the tonoplast in pineapple [10] but localisation and substrate specificity of the other exporters described need to be confirmed.

The presence of sugar importers and exporters in the pineapple tonoplast demands regulatory mechanisms which curtail futile cycling of sugars in and out of the vacuole during the day. Low transcript abundances of *SUC4*, *ERDL6* and *SWEET16* compared to the *TSTs* (Table 1) imply low activity of the putative sugar exporters in pineapple which could curtail futile cycling at times when *TST* is active [45,46]. In pineapple, *TST* transcript abundance peaks at the end of the night and into the first hours of the photoperiod, suggesting some level of diel transcriptional control (Table 1). This, together with possible activation of *TST* via post-translational phosphorylation, as reported for *Arabidopsis* [48], could be hypothesized as a mechanism for ensuring vacuolar uptake of sucrose occurs during the day, with minimal efflux of hexoses to the cytosol. Whether or not the putative sugar exporters are also subject to transcriptional and post-translational regulation to curtail day-time export and/or enhance nocturnal export needs to be tested.

Conclusions

The provision of PEP is a limiting factor for nocturnal CO₂ uptake in CAM plants and represents a significant sink for carbohydrate. Experimental evidence suggests that starch-storing CAM plants balance the sink demands of CAM with growth and maintenance via a re-routing of C₃ starch degradative pathways and diversification in chloroplastic transporters, some of which are under circadian control. In soluble sugar-storing CAM species, it remains to be established if vacuolar sugar transporters have diversified from those in C₃ plants in terms of function and/or regulation, perhaps via the acquisition of circadian associated cis-regulatory elements, as indicated for several CAM pathway genes [38]. Further development of diel flux balance models [26,49] will be critical for revealing the potential contribution and energetic implications of alternative pathways for carbohydrate processing. Such knowledge could help to identify the minimum set of genetic interventions required to engineer CAM into C₃ plants as a means of improving water use efficiency [2,50] whilst minimising detrimental consequences for growth.

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Transporter	<i>Arabidopsis</i> gene locus	Gene name	<i>A.comosus</i> ortholog (RPKM)*	Diel transcript abundance
Plastidic glc-6-P/P translocator 1	At5g54800	<i>GPT1</i>	Aco014864.1 (87228.5)	
Plastidic glc-6-P/P translocator 2	At1g61800	<i>GPT2</i>	Aco009063.1 (1785.2)	
Chloroplast glucose transporter	At5g16150	<i>pGLC</i>	Aco026982.1 (1510.7)	
Chloroplast maltose transporter	At5g17520	<i>MEX1</i>	Aco017242.1 (344.9)	
Tonoplast sugar transporter 1; vacuolar sugar/monosaccharide importer, Proton antiporter	At1g20840	<i>TST1</i>	Aco015779.1 (13145.2)	
Tonoplast sugar transporter 2; vacuolar sugar/monosaccharide importer, Proton antiporter	At4g35300	<i>TST2</i>	Aco011916.1 (1985.7)	
Vacuolar ERD6-like 6; glucose exporter Proton symporter	At1g75220	<i>ERDL6</i>	Aco006192.1 (32.7)	
Vacuolar sucrose exporter Proton symporter	At1g09960	<i>SUC4</i>	Aco000269.1 (5.0)	
Vacuolar glc/fru/suc exporter Facilitated diffusion	At3g16690	<i>SWEET16</i>	Aco002476.1 (54.5)	

Table 1. Putative orthologues of chloroplast and vacuolar sugar transporters implicated in the diel processing of carbohydrate in pineapple leaves.

Orthologues of *Arabidopsis thaliana* chloroplastic and vacuolar metabolite sugar transporters were identified from the pineapple (*Ananas comosus*) genome [34]. RNA-seq data were collected from green leaves sampled at 13 time-points over a 24 h light/dark cycle. Maximum transcript abundances for each orthologue in pineapple leaves were calculated as RPKM*. Diel patterns of transcript abundance over the light/dark cycle were calculated from FPKM values which were converted to z-scores with a blue-black-yellow representation in the heat maps, where blue is lowest, yellow is the highest and black is the median FPKM values, respectively.

Table 2. Activities of key enzymes implicated in the hydrolytic and phosphorolytic routes of starch degradation in *Arabidopsis* and *Mesembryanthemum crystallinum*

Enzyme activities are shown in relation to the measured rate of nocturnal starch degradation in leaves of *A.thaliana* and in *M. crystallinum* in C₃ or CAM mode, the latter induced by watering with 500 mM NaCl for 2 weeks. Measured activities are expressed as nmol min⁻¹ g⁻¹ fwt and values shown are the mean ± SE from 3 biological replicates. Asterisks mark activities in CAM-performing *M. crystallinum* that are significantly different from C₃ performing *M. crystallinum* (P ≤ 0.05). All plants were grown and sampled under identical growing conditions (12 h photoperiod, 25/19 °C day/night temperature, photon flux density at plant height of 350 μmol m⁻² s⁻¹).

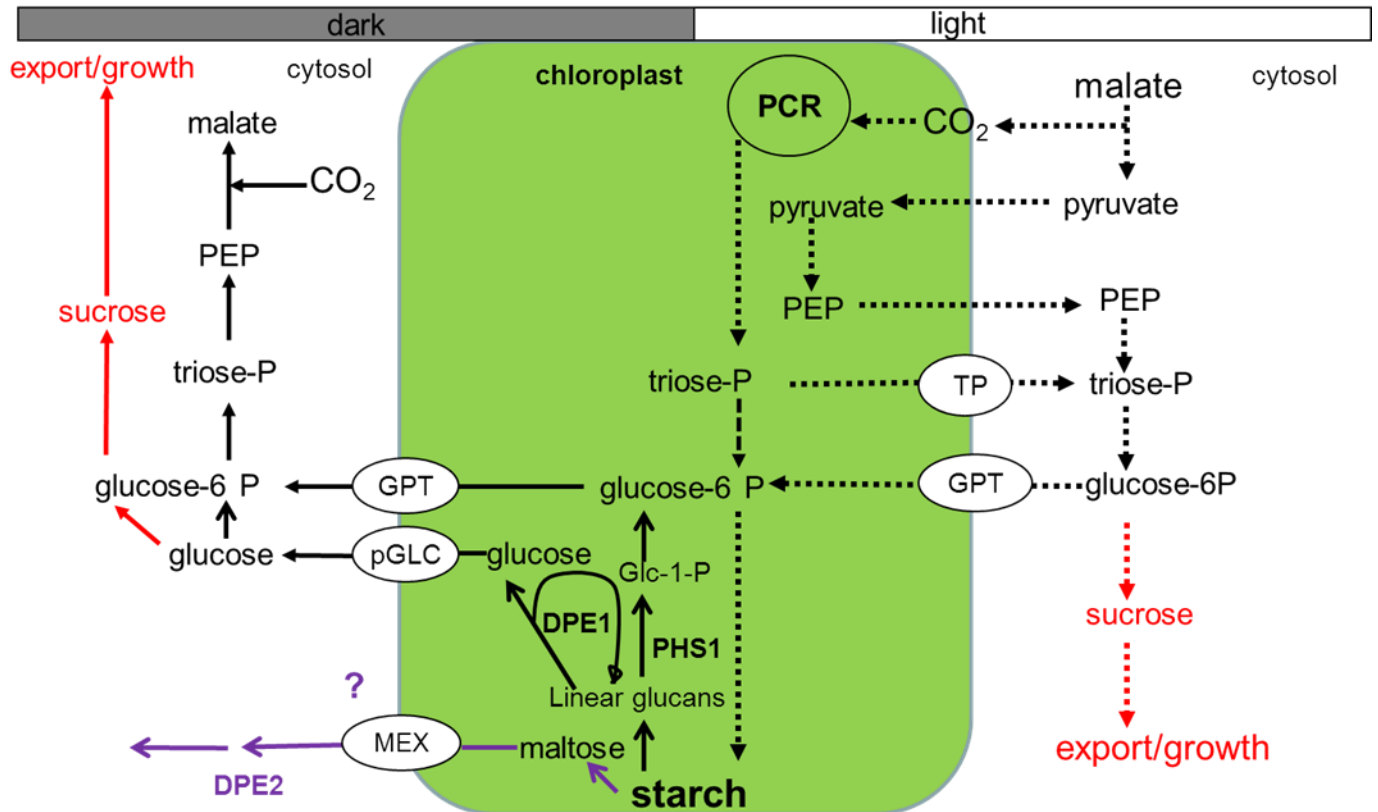
	<i>Arabidopsis thaliana</i>	<i>M. crystallinum</i> C ₃	<i>M. crystallinum</i> CAM
D-enzyme (DPE1) (chloroplastic)	260.1 ± 7	1300.3 ± 50	2256.2 ± 61*
DPE-2 (cytosolic)	147.6 ± 10	22.1 ± 2	34.0 ± 3
α-glucan phosphorylase (PHS)	75.0 ± 5	107.4 ± 8	194.5 ± 7*
Nocturnal starch degradation	102 ± 7	53 ± 5	88 ± 7*

Figure legend

Figure 1. Summary outline of pathways and transporters implicated in the diel processing of carbohydrates in CAM.

Proposed flow of carbon and intercellular transport processes for the day/night processing of metabolites and sugars in; a) a starch-storing CAM plant and b) a soluble-sugar storing CAM plant. Day-time fluxes that include those through the photosynthetic carbon reduction (PCR) cycle are shown by the dotted lines and night-time fluxes by the solid lines. Transporters shown within the white circles are; triose-P/P translocator (TP), glucose-6-P translocator (GPT), plastidic glucose transporter (pGLC), maltose transporter (MEX). Also shown are metabolic steps implicated in nocturnal processing of starch that are catalysed by chloroplastic α -glucan phosphorylase (PHS1), chloroplastic disproportionating enzyme (DPE1) and cytosolic disproportionating enzyme (DPE2). Steps implicated in the nocturnal processing of maltose are shown in purple; their significance for carbohydrate metabolism in CAM species is still to be established. In soluble sugar-storing CAM plants, the molecular identity of transporters responsible for the day-time import of sucrose and nocturnal export of hexoses are still to be conclusively identified.

a) Starch storing CAM plants



b) Soluble sugar storing CAM plants

